

Review

Gelatin structure and composition linked to hard capsule dissolution: A review



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ABSTRACT

Gelatin obtained from pig skin constitutes about 50% of world production and is mainly composed of collagen extracted from skin by acidic baths and thermal treatments. The gelatin is used to make various products, notably hard gelatin capsules (HGC) which of varying solubility in water. This issue has been known for many years and has been, and remains, a subject of study and debate. The main reason for low gelatin dissolution rates is its tendency to form cross-links in the denatured collagen chains under specific conditions which stabilize the gel network and prevent dissolution. As it is extracted from animal tissues, gelatin may contain molecules other than collagen (sugars, lipids and other proteins) which may react with collagen chains to form covalent bonds. Although this biopolymer has been the subject of numerous publications, its structure and composition is not well defined. Indeed, there are many differences from an article to another. Consequently, the causes of HGC dissolution are not well identified and controlled.

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1. Introduction

Gelatin derived from animal tissue has been known since antiquity and was first used as glue as far back as 6000 BC. During the 16th century, at the court of Henry VIII of England, gelatin was an ingredient of dishes at every banquet. Over time, its manufacture became industrialized and its applications have increased in number (Schrieber & Gareis, 2007). It is now widely used in the food, photographic and pharmaceutical industries.

The most abundant sources of gelatin production are pig skin (46%), bovine hides (29.4%) and pig and cattle bones (23.1%). Fish gelatin represented less than 1.5% of total gelatin production in 2007 (Gomez-Guillén et al., 2009). In this review we focus on the most abundant part of the production, i.e. pig skin gelatin, taking account of knowledge on all gelatin origins.

This biopolymer consists of proteins (85–92%), mineral salts and water. It is produced by the partial hydrolysis of collagen (Schrieber & Gareis, 2007). Depending on the raw material used (source and age of the animal), collagen does not have exactly the same structure, composition and properties, and gelatin does not either. Indeed, 28 different types of collagen have been identified (Ricard-Blum, 2010). During the gelatin-making process, proteins are extracted from skin and bone by acid or alkaline baths and thermal pre-treatments. A thermal process is then used to separate proteins from the rest of the raw material (Schrieber & Gareis, 2007). Depending on the manufacturing process, the extract is then deionized, sterilized and dried, but more steps can be added. The dried matter obtained is called gelatin. There are two types of gelatin, A and B, produced from acid and alkaline pre-treatments, respectively.

Gelatin is used as the main ingredient of the hard capsules used in the pharmaceutical industry. An important property of these hard capsules is that they melt in water at a temperature above 30 °C and easily release drugs they contain in the human digestive tract due to temperature, gastric pH and digestive enzymes. However, to be sold on the market, a hard capsule has to pass the dissolution test in water according to the specifications in the United States Pharmacopeia 711 harmonized with the corresponding texts of the European Pharmacopoeia and the Japanese Pharmacopoeia (U. S. Pharmacopeial Convention, 2012). Sometimes gelatin hard capsules present an insufficient dissolution rate in water. This dissolution issue has been known since 1974 and was revealed by studying chloramphenicol capsules (Khalil, Ali, & Abdel Khalek, 1974). Since then, many publications have dealt with the gelatin dissolution and shown that this issue is still a concern. The main cause of this poor dissolution is the tendency of gelatin to form cross-links in high relative humidity and temperature conditions or in the presence of chemical compounds such as aldehydes (Ofner, Zhang, Jobeck, & Bowman, 2001). In gastric fluids, cross-linked hard capsules can be dissolved easily in the same way as non cross-linked hard capsules (Meyer et al., 2000). This observation led to the modification of the United-States Pharmacopeia monograph on gelatin capsule dissolution testing in which the use of enzymes in dissolution media is allowed in some circumstances, i.e. in the two-tier test (Cole, Cad, & Benameur, 2008). However, specifications vary according to the pharmacopeia and water is still generally commonly used as a dissolution medium

(Chiwele, Jones, & Podczek, 2000). An alternative to gelatin was developed in the industry with other polymers like HPMC (Hydroxypropyl methylcellulose) most likely to replace gelatin (Al-Tabakha, 2010). However, the properties of HPMC are different from those of gelatin and hard gelatin capsules (HGC) are still the second most used form of oral dosage after tablets (around 70% for tablets and around 28% for HGC in 2000) and the trend is increasing, showing that other polymers are not about to replace gelatin in hard capsules (Stegemann, 2002).

Cross-link formation depends on many parameters. The main difficulty is to order the factors affecting cross-links according to their nature and impact on dissolution. The raw material used plays an important role in the degree of cross-linking. Indeed, in young animals, collagen molecules present few cross-links which confer elasticity to skin. But with aging, more cross-links are found in the collagen network, forming an extremely stable structure. The gelatin-making process also influences the cross-link degree, more particularly during acid or alkaline pre-treatments which partly cleave collagen cross-links to give a denatured collagen structure (Schrieber & Gareis, 2007).

The raw material and the manufacturing process may play an important role in the dissolution rate of HGC, but other factors like the presence of various reactive compounds have to be taken in account. Indeed, there are many different molecules in pig skin and, despite pre-treatment and thermal extraction, the extract may contain not only denatured collagens but also other extracellular matrix components such as proteoglycans, elastin or fibronectin which interact with collagen in connective tissue. The latter molecules may create cross-links with the denatured collagens and reduce gelatin dissolution. Moreover, sugars or lipids may also be extracted from the raw material during the manufacturing process and be involved in cross-link formation.

The aim of this review is to provide a state of the art on knowledge of gelatin and identify the factors affecting gelatin dissolution. Thus it aims at contributing to better understanding of this issue and providing an overview of research in this field.

2. Collagen composition and structure

Skin is mainly composed of type I collagen and, to a lesser extent, type III collagen (Bruckner, 2010) (see Table 1). Collagen is composed of three α chains forming a triple-helix structure. The α -chain consists of continuous repetitions of Gly-X-Y amino acid sequences where X is mostly proline and Y is mostly hydroxyproline (Bailey & Light, 1989). The latter amino acid is specific to the collagen molecule (Hofman, Hall, Cleaver, & Marshall, 2011). Because of this primary sequence full of proline and hydroxyproline residues, which are regularly located in the α -chain in the motif Gly-Pro-Hyp, the α -chain adopts a left-handed helix type conformation which is unstable in individual state. Indeed, proline and hydroxyproline have rings which force the chain to form a helix due to steric hindrance (Okuyama, Miyama, Mizuno, & Bachinger, 2012). When three chains are linked together they form a very stable right-handed triple helix (Bailey & Light, 1989). This triple-helix is stabilized by intra and inter-chain hydrogen bonds. In this dense structure, glycine residues are oriented in the center while

Table 1
Collagen types and their localizations in animals.

Collagen types/structural organization		Localization	Abundance in skin ^a	References
I	FFC	Skin, intra muscular, tendon, bone, dentine, cornea	80–85%	(Bailey & Light, 1989), (Brinckmann, Notbohm, & Müller, 2005; Riekkki et al., 2002)
II	FFC	Cartilage, disc, vitreous humour	–	(Bailey & Light, 1989), (Brinckmann et al., 2005; Riekkki et al., 2002)
III	FFC	Skin, intramuscular, vascular, intestine, vessel, uterus	10–15%	(Bailey & Light, 1989), (Brinckmann et al., 2005; Riekkki et al., 2002)
IV	NFC	Neuromuscular junction, basement membranes	–	(Bailey & Light, 1989; Fox, 2008)
V	FFC	Skin, intramuscular, embryonic tissues, cornea, bone	2–4%	(Bailey & Light, 1989), (Brinckmann et al., 2005; Smith, Holbrook, & Madri, 1986)
VI	BFFC	Skin (epidermis), vascular system, bone, cartilage, cornea	n/a ^b	(Soderhall et al., 2007), (Bailey & Light, 1989), (Brinckmann et al., 2005)
VII	BFFC	Skin, amniotic membrane, bladder, oral mucosa, umbilical cord, amnion	0.001%	(Bailey & Light, 1989; Brinckmann et al., 2005; Brucknertuderman, Schnyder, Winterhalter, & Bruckner, 1987; Chagnot et al., 2012)
VIII	Network	Skin, basement membranes, descemet's membrane, vessel, bone, brain, heart, kidney, cartilage	n/a	(Brinckmann et al., 2005; Suttmüller, Bruijn, & De Heer, 1997)
IX	FACIT	Articular cartilage, cornea, vitreous	–	(Brinckmann et al., 2005; Martel-Pelletier, Boileau, Pelletier, & Roughley, 2008)
X	NFC	Hypertrophic cartilage	–	(Sweeney, Roberts, Corbo, & Jacenko, 2010)
XI	FFC	Cartilage, intervertebral disc	–	(Bailey & Light, 1989; Brinckmann et al., 2005)
XII	FACIT	Skin, tendon, cartilage	n/a	(Brinckmann et al., 2005)
XIII	MACIT	Skin, bone, neuronal structures, endothelial cells, heart, eye, skeletal muscle	n/a	(Brinckmann et al., 2005; Seppanen et al., 2006; Ylonen et al., 2005)
XIV	FACIT	Skin, vessel, bone, cartilage, eye, nerve, tendon, uterus	n/a	(Brinckmann et al., 2005)
XV	MPC	Skin, capillaries, placenta, kidney, heart, ovary, testis	n/a	(Brinckmann et al., 2005)
XVI	FACIT	Skin, heart, kidney, smooth muscle	n/a	(Brinckmann et al., 2005)
XVII	MACIT	Hemidesmosome in epithelia, neuronal structures	–	(Brinckmann et al., 2005; Has & Kern, 2010; Seppanen et al., 2006)
XVIII	MPC	Perivascular basement membrane, kidney, liver, lung	–	(Brinckmann et al., 2005)
XIX	FACIT	Skin, central neurons, basement membrane zone in skeletal muscle, spleen, prostate, kidney, liver, placenta, colon	n/a	(Brinckmann et al., 2005; Chagnot et al., 2012; Su, Gorse, Ramirez, & Fox, 2010)
XX	FACIT	Corneal epithelium (chick)	–	(Brinckmann et al., 2005; Chagnot et al., 2012)
XXI	FACIT	extracellular matrix of the blood vessel walls, vessel, heart, stomach, kidney, skeletal muscle, placenta	–	(Brinckmann et al., 2005; Chagnot et al., 2012; Chou & Li, 2002)
XXII	FACIT	Tissue junctions	–	(Chagnot et al., 2012; Koch et al., 2004)
XXIII	MACIT	Prostate cancer and distant metastases, heart, retina	–	(Banyard et al., 2007), (Brinckmann et al., 2005)
XXIV	FFC	Bone, cornea	–	(Brinckmann et al., 2005; Chagnot et al., 2012; Matsuo et al., 2008)
XXV	MACIT	Neuronal structures, brain, heart, testis, eye	–	(Brinckmann et al., 2005; Hashimoto et al., 2002)
XXVI	BFFC	Testis and ovary	–	(Chagnot et al., 2012; Sato et al., 2002)
XXVII	FFC	Adult cartilage	–	(Chagnot et al., 2012; Hjorten et al., 2007)
XXVIII	BFFC	Neuronal tissue	–	(Chagnot et al., 2012; Grimal et al., 2010)

^a The abundance of collagen type depends on the age of animals and species. The values given in the table correspond to a human adult.

^b n/a: no answer.

the side chains of the X and Y residues are exposed to solvent (Vaca Chavez, Hellstrand, & Halle, 2006). At the extremity of the triple-helices, the collagen chains form non-helical structures called the telopeptide regions.

Collagens can have diverse supramolecular organizations, namely fibril-forming collagen (FFC), network-forming collagen (NFC), beaded filament-forming collagen (BFFC), membrane associated collagens with interrupted triple-helices (MACIT), fibril-associated collagens with interrupted triple helices (FACIT) and multiplexins (MPC) (Chagnot, Listrat, Astruc, & Desvaux, 2012). The most abundant molecular structure in skin is FFC while FACIT, MACIT, MPC and BFFC are present in lower proportions. Other molecules like elastin, proteoglycans, laminin or fibronectin are linked together, forming a network with collagens in the extracellular matrix (Fig. 1). In the case of fibrils forming collagen, the triple-helix structures are linked together by covalent bonds to form a fibril that links to other fibrils to form a collagen fiber (Schrieber & Gareis, 2007). Collagen presents different cross-linkage levels as a function of the type of tissue and the age of the animals. Indeed, in dense tissues like bones, collagen is more

cross-linked than in loose tissues, making the matrix more rigid. Likewise, collagen is more cross-linked in old than in young animals, reducing, for instance, skin elasticity (Eyre & Wu, 2005; Schrieber & Gareis, 2007; Shoulders & Raines, 2009).

3. Gelatin composition

As gelatin contains a majority of denatured collagens, its amino acid composition is close to that of collagen molecules. However, some variations remain due to the manufacturing process and the molecular organization of gelatin is very different from that of native collagen. The transformation of collagen to gelatin leads to changes in the molecular composition of several aminoacids. Indeed, the alkaline process deaminates glutamine into glutamic acid and asparagine into aspartic acid. Thus the proportion of aspartic acid and glutamic acid is higher in type B gelatin than in type A (Singh, Manikandan, Venugopal, Rama Rao, 2002; Taheri, bedian Kenari, Gildberg, & Behnam, 2009; Zhou & Regenstien, 2006).

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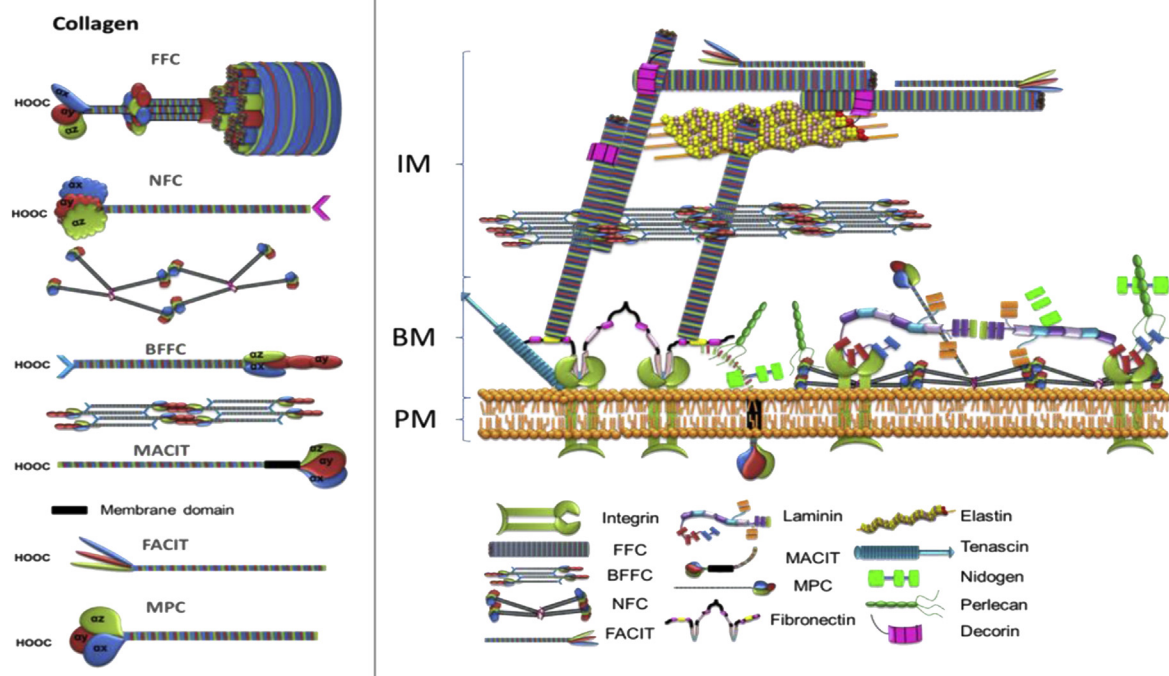


Fig. 1. Schematic representation of collagens structures (left), supramolecular organizations and their interactions with the extracellular matrix components (right) (PM: Plasma membrane; BM: Basement membrane; IM: Interstitial matrix) (Chagnot et al., 2012).

The amino acid composition of gelatin is not clearly defined. Indeed, in mammalian gelatins, proline and hydroxyproline represent about 30% of total aminoacids in the study of [Muyonga, Cole, and Duodu \(2004\)](#) while this proportion was 23% in the work of [Farris, Song, and Huang \(2009\)](#), as shown in [Table 2](#).

Moreover, [Farris et al. \(2009\)](#) did not find cysteine in gelatin from pig skin although [Bailey and Light \(1989\)](#) reported its presence in type III collagen. Although there is no information in literature about the abundance of this collagen in pig skin, it may represent a significant part of the total collagen as, in human skin, it makes up about 15% of the total collagen. In addition to proteins, the raw material contains sugars, lipids, small molecules and ions naturally present in bones and skin. Despite all the purification steps, gelatin may still contain traces of sugars, lipids and salts. These molecules interact with the proteic fibers of gelatin and can form covalent bonds (see part 4.2.4). Some reactions with sugars like Maillard reactions are the cause of the brown color of gelatin during the extraction steps and can modify gel properties ([Rbii, 2010](#)). Small peptides formed from collagen during the process are also found in gelatin. Thus, according to the manufacturing process and the raw material, the quantity of small peptides

present in gelatin changes as a function of the change in distribution of molecular weight from one gelatin to another ([Elharfaoui, Djabourov, & Babel, 2007](#)).

4. Gelatin structure and gelation mechanism

4.1. Gelatin structure

During the gelatin manufacturing process, collagen is denatured and loses its native structure. The collagen fibers forming helices lose their conformation during heating and partially recover their structure during cooling. Water is trapped in the mesh of chains and the gelatin forms a gel. The gelatin structure is different to that of collagen because the helices are partially reformed.

The structure of gelatin changes during gelation. Indeed, according to the state of the gel, the chains have different space arrangements and different interactions. These two characteristics depend on the gelatin concentration, temperature and the energy necessary for the formation of the secondary structure. One double strand structure can be formed by two α -chains or by one α -chain

Table 2

Amino acids composition of the pig skin gelatin from results of [Farris et al., \(2009\)](#).

Amino acid	Percentage	Amino acid	Percentage
Glycine	32.20	Threonine	1.80
Proline	13.10	Phenylalanine	1.38
Alanine	11.05	Isoleucine	1.02
Hydroxyproline	9.80	Hydroxylysine	0.75
Glutamic acid	7.10	Asparagine	0.60
Arginine	4.96	Histidine	0.45
Aspartic acid	4.42	Tyrosine	0.35
Serine	3.40	Methionine	0.32
Lysine	2.65	Tryptophan	—
Leucine	2.35	Cysteine	—
Valine	1.90		

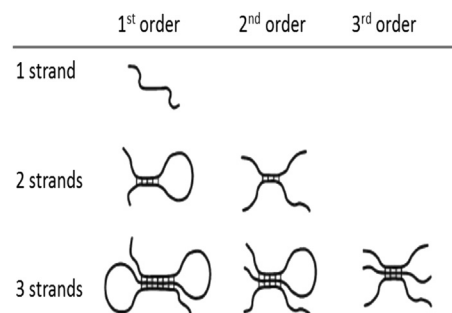


Fig. 2. The different types of chains organizations in gelatin. Reprinted with permission from ([Guo et al., 2003](#)) Copyright (2003) American Chemical Society.

which creates a loop. Likewise, a triple strand structure can be formed by three different α -chains, or by two α -chains one of which forms a loop, or by only one α -chain with two loops (Fig. 2) (Guo, Colby, Lusignan, & Whitesides, 2003).

Guo et al. (2003) developed three different reaction orders for a gelatin from limed bones (species unknown). The first order means that there is only one α -chain in the structure. The second one is used to present reactions with two different α -chains and the third order includes three different α -chains. The structures formed are reversible and stable only if they have a minimum length, indicating that these helices are stabilized by weak bonds. For instance, if a helix is too small, it is easily melt. According to the authors, the length of a helix with one loop has to be twice the length of a helix with no loop to be stable. Indeed, the formation of structures with loops requires more energy than those without loops.

According to Coppola, Djabourov, and Ferrand (2012), who characterized the gelatin structure by Differential Scanning Calorimetry (DSC), a type B bovine hide gelatin film can have three different structural states: the amorphous state corresponds to a coil structure with primary chains, the semi-crystallized state is composed of triple-helices and a coil structure, and the crystalized state corresponds to the packing of triple-helices and a coil structure (Fig. 3). This description agrees with those of the studies of Harrington and Rao (1970) and Oakenfull and Scott (2003) who identified junction zones in the gelatin structure composed by individual triple-helices or by triple-helices aggregated together. Harrington and Rao (1970) observed these junction zones in pyrrolidine-rich regions containing large amounts of Pro and Hyp amino acids.

These three different states depend on the drying speed of gelatin films. The amorphous state is obtained when gelatin films are dried quickly, whereas the crystalized state corresponds to a slow drying rate (Coppola et al., 2012). This observation has been also made by Jones (2004) who saw that the thickness of films

affected the speed of drying and had an impact on the space arrangement of the molecules.

Gelatin structure changes with the humidity rate, temperature, concentration and content of various substances in gelatin (Coppola et al., 2012; Jones, 2004; Kozlov & Burdygina, 1983). Elharfaoui et al., (2007) found that the nucleation of gelatin chains from limed beef bones is very sensitive to gelatin concentration during the cooling step. The amount of helix structures in gelatin increases as concentration increases.

The structure is also influenced by the molecular weight distribution of gelatin chains. Collagen α -chains have a well-defined molecular weight (around 110 000 g/mole). In gelatin, these α -chains form β and γ chains with molecular weights of 200 000 g/mole and 300 000 g/mole, respectively. The β and γ chains are the result of α -chains linked together by covalent bonds which are different from the double or triple strand structures described by Guo et al. (2003) forming helices stabilized by weak bonds. Indeed, two α -chains covalently linked together form a double strand structure named β -chain, and three α -chains can form a triple helix named γ -chain also stabilized by covalent bonds (Diaz, Lopez, Matiacevich, Osorio, & Enrione, 2011; Gomez-Guillén, Giménez, Lopez-Caballero, Montero, 2011; Stainsby, 1987; Taheri et al., 2009). Structures of higher molecular weight are found in gelatin and are called microgels according to Elharfaoui et al. (2007) although these structures do not form a gel *sensu stricto*.

The molecular weight distribution depends on the gelatin manufacturing process which causes molecular degradation (Elharfaoui et al., 2007). The raw material used also influences the range of molecular weights in finished products. Indeed, bone and skin collagen from bovine, porcine and fish will give different molecular weight distributions. The age of the animals used also has an influence because the collagen of older animals has more cross-links than that of young animals. According to Elharfaoui

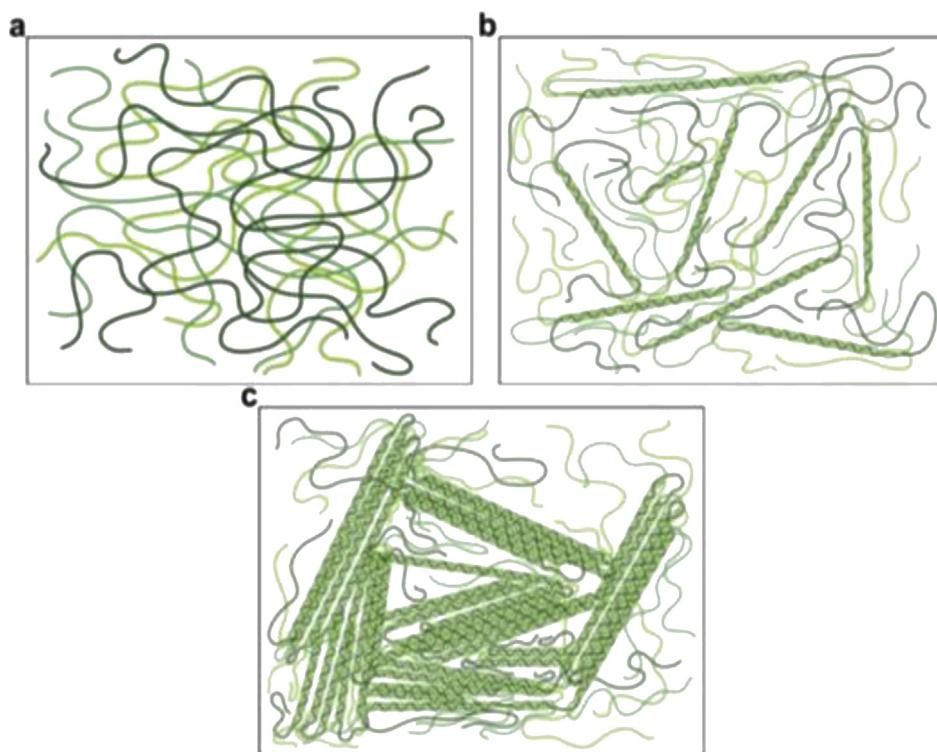


Fig. 3. Scheme of films gelatin structure. a) amorphous coils; b) triple helices and coils; c) bundles of triple helices and coils (Coppola et al., 2012).

et al. (2007), this cross-linked collagen gives gelatin with high molecular weight compounds. These high molecular weights are highly branched, non linear chains and hinder helix renaturation. A large number of sub-units of α -chains, of low molecular weight, also hinders helix renaturation because these sub-units are too small to create entanglements, preventing triple-helix nucleation.

As the gelatin structure depends on the origin of the raw material, it is possible that the structure of type A pig skin gelatin differs from the structure described here, since all these observations have been made on type B bovine hide or bone gelatin. However these studies give important information on the factors influencing gelatin structure and how they induce changes in the polymer.

Finally, according to the studies cited above the structure of gelatin presents a high degree of complexity and is influenced by many factors. It is stabilized by different types of covalent bonds and its modularity is made possible by numerous weak interactions.

4.2. Nature of interactions

4.2.1. Hydrogen bonds

Double strand or triple helix structures are stabilized by hydrogen bonds formed by glycine residues placed in the α -chain every three amino acids (Ramachandran & Kartha, 1954). These numerous interactions are situated regularly on the chain and maintain the triple-helical structure. The hydrogen atoms of glycine are located inside the triple helix and form a weak bond with the oxygen atom of the carboxyl groups (Fig. 4, a) (Bailey & Light, 1989; Guo et al., 2003; Oakenfull & Scott, 2003).

Solvent water molecules are also involved in the hydrogen bonds of the gelatin network. Oakenfull and Scott (2003), who studied gelatin gels in deuterium oxide, observed that the three-dimensional gelatin structure is stabilized by both $-NH$ groups of one gelatin chain forming a hydrogen bond with the $-CO$ groups of another chain and the hydrogen bonds formed by water molecules with gelatin chains. This mechanism was previously hypothesized by Traub and Piez (1971), though without precision, on the interaction mechanisms of water molecules and gelatin residues.

Hydroxyproline would stabilize the triple helical areas (the junction zones) forming H-bonded water chains, bridging the OH group of Hydroxyproline of one strand with the backbone $-CO$ of

the same or another strand, however this view has been contested (Vaca Chavez et al., 2006). The implication of Hyp residues in hydrogen bonds was also investigated by Bailey and Light (1989). Water molecules would form hydrogen bonds between the hydroxyl groups of two hydroxyproline residues or between the hydroxyl group of hydroxyproline and the $-CO$ group of the gelatin backbone (Bailey & Light, 1989) (Fig. 4, b).

To summarise, hydrogen bonds can be of several types: either direct between the $-CO$ group and hydrogen of glycine residue belonging to two adjacent backbones, or between $-NH$ groups of a chain and $-CO$ groups of another chain, or via water molecules bridging $-CO$ and $-OH$ groups of hydroxyproline, or water molecules bridging $-OH$ groups of two hydroxyproline residues. Whatever the case, the number and type of hydrogen bonds in gelatin structure are not clearly defined.

4.2.2. Hydrophobic interactions

In 1995, scientists studied the assembly of collagen molecules and demonstrated that hydrophobic bonds play an insignificant role in triple-helix formation (Leikin, Rau, & Parsegian, 1995). However, these interactions could have a major effect on β -sheet structure formation according to Xu, Li, Tang, Qiao, and Jiang (2012) who showed that aggregate formation in pig skin gelatin grafted with glycidol increased with concentration and that hydrophobic bonds may play an important role in this phenomenon. Using UV analysis, they demonstrated that hydrophobic interactions increased and competed with hydrogen bonding as a function of increasing gelatin concentration. Hydrophobic interactions, as nonspecific interactions, are the major driving force for protein folding and possibly cause chain aggregation. The strength of electrostatic repulsion between charged residues is enhanced in the hydrophobic region and favors the formation of a β -sheet structure, causing the extension of molecular chains. Moreover, Xu et al. (2012) have used Circular Dichroism analysis to demonstrate that the β -sheet structure increased with increasing hydrophobicity, but the authors used a gelatin sample containing glycidol compounds and not a pure gelatin. Eyre and Wu (2005) revealed that strong hydrophobic interactions occurred between the C-terminal globular domains in the network of collagen types VIII and X, knowing that type VIII collagen is found in skin (see Table 1).

Gelatin has tensioactive properties and acts as a surfactant. These properties are used in food and pharmaceutical industries to

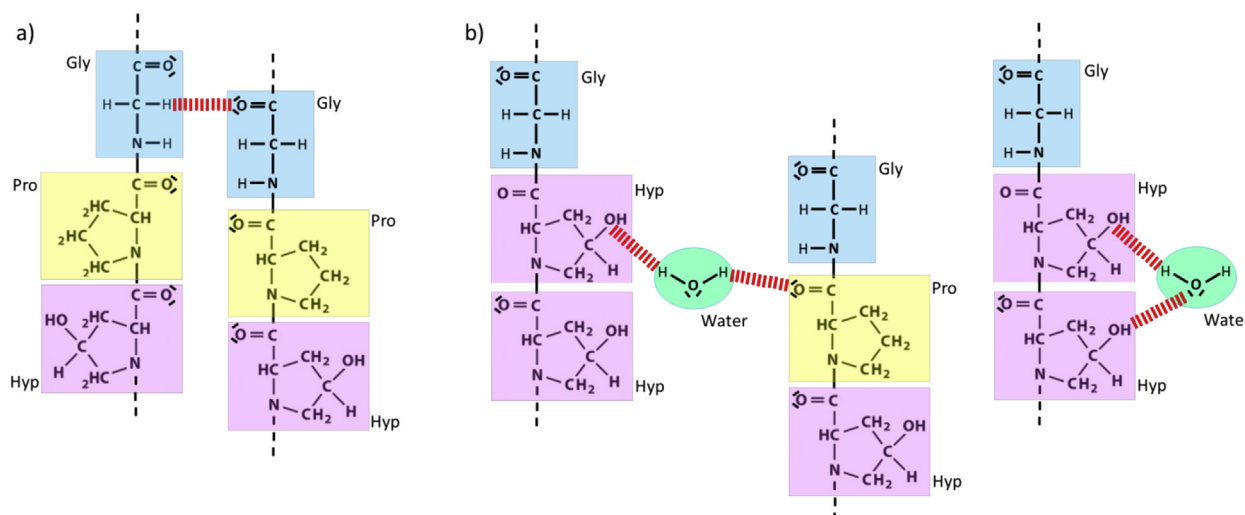
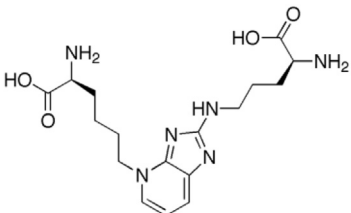
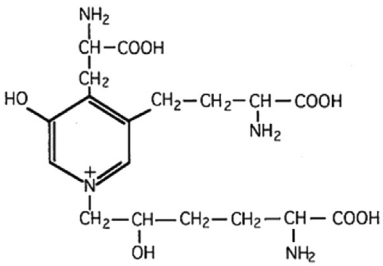
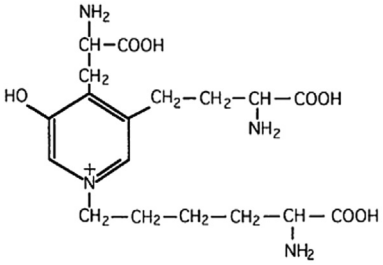


Fig. 4. Examples of hydrogen bond (dotted line) in gelatin chains (a) and between gelatin chains and water molecules (b).

Table 3

Schemes of fluorescent cross-links in gelatin; pentosidine, pyridinoline and deoxypyridinoline.

Cross-link	Molecular representation	References
Pentosidine		(Ricard - Blum, 2010)
Pyridinoline		(Samma et al., 1996)
Deoxypyridinoline		

stabilize foams or emulsions. According to Lin, Wu, and Tsao (2003), in solutions 7% of the amino acids in the gelatin structure are strongly hydrophobic and create hydrophobic interactions at the interface air/water.

However, the proportion of hydrophobic interactions in gelatin structure remains rather difficult to establish due to the lack of knowledge on the subject and significant publications.

4.2.3. Electrostatic interactions

As 85–92% of gelatin is composed of proteins, it contains both cationic and anionic groups. The electrostatic interactions in this polyelectrolyte gel are influenced by pH and salt concentrations. Yang et al. (1997), studied the swelling behavior of gelatin using solutions with different NaCl concentrations. They observed that the degree of swelling was influenced by the degree of ionization of the solution and attributed this to the formation of ion pairs between network charges and counterions. This observation agrees with the results of Klooster, Vandertouw, and Mandel (1984) who highlighted the influence of ion-pairs on the conformation of polyelectrolyte chains in solution. Yang et al. (1997) measured the shear modulus of gelatin gels and demonstrated that the formation of ion-pairs led to cross-linking in gelatin because of their aggregation. The increase of ionization increased the ion pair cross-linking with consequences on shear modulus. These authors noticed that the shear modulus was higher for an anionic gel than for a cationic one and attributed it to the greater facility to trigger electrostatic interaction between COO^- (of the gelatin) and Na^+ (in solution) than between the NH_3^+ groups of gelatin and Cl^- .

Miyawaki et al., (2003) investigated the effect of water potential on sol–gel transition and the intermolecular interactions of pig skin gelatin. They suggested that during gelation, triple helix formation involved electrostatic and hydrophobic interactions, as well as hydrogen bonding.

The addition of salt can modify electrostatic interactions and affect the stabilization of the gelatin network. Haug, Draget, and Smidsrød (2003), measuring the mechanical properties of A-type fish gelatin with varying pH and salt concentration, concluded that electrostatic interactions may contribute to the stabilization of the junction zones in gelatin. On the contrary, using light scattering Bohidar and Maity (1998) pointed out that different concentrations of NaCl did not change the degree of helicity in gelatin. They considered that electrostatic interactions did not play an active role in gelation. However, the raw material used was not specified.

4.2.4. Covalent bonds

Despite chemical and thermal treatments, covalent bonds (or cross-links) found in collagen could also be found in gelatin and influence its mechanical properties. The collagen presents various cross-links; for instance, in skin, type III, VI, VII and XVI collagens can form disulfide bonds and type I, III, V and VII collagens can form N(γ -glutamyl)lysine isopeptide. This cross-link is naturally formed by transglutaminase-2 between glutamine and lysine amino acids (Eyre & Wu, 2005; Sjöberg & Bulterjys, 2009). Table 4 gives the list of all the cross-links potentially found in skin collagen and/or in gelatin.

In a first part, the covalent bonds found in collagen are discussed. Baynes and Dominiczak (2004) explained that in collagen, other covalent bonds are formed by the allysine pathway (Fig. 5). Two allysine residues (lysine with aldehyde group) undergo aldolic condensation to create a cross-link. Another possible reaction includes both allysine and lysine residues, forming a Schiff base to give a lysinorleucine. Eyre and Wu (2005) described not only the allysine pathway (Fig. 6) but also the hydroxyallysine pathway (Fig. 7). The latter is found in bone tissue whereas the former is located in skin. The authors identified lysine and hydroxylysine as precursors of cross-links formation. Both these amino acids are used by Lysyl oxidase to form divalent or trivalent cross-links whether the cross-link implies two or three collagen strands, respectively. In skin, the divalent cross-links are hydroxylysinonorleucines (glycosylated or not) and intra molecular dimers not described by the authors. Trivalent cross-links correspond to hystidinyl hydroxylysinonorleucines (HHL) formed with a helix with histidine and a divalent hydroxylysinonorleucine glycosylated cross-link. Trivalent cross-links resulting from non-glycosylated divalent hydroxylysinonorleucine have not been identified. Cross-links are present in the same type of collagen but also between different types. Indeed, Edman N-terminal analysis of cross-linked peptides obtained after digestion of collagens revealed cross-links between collagen type I and III. It seems that cross-links are more tissue specific than collagen type specific (Eyre & Wu, 2005).

Pentosidine is a cross-link naturally found in protein from skin, including collagen (Sell et al., 1991; Vos et al., 2013). This advanced glycosylation end-product results from the reaction between pentoses and arginine or lysine side chain. Hexoses also contribute to the formation of pentosidine by sugar fragmentation during the long-term glycosylation of proteins. Sell et al. (1991) describe the pentosidine formation mechanism as the dehydration of the pentose-derived Amadori compound which leads to an intermediate product. The latter is attacked by the guanido group of an arginine residue. It has not been established whether this mechanism necessarily requires the Amadori rearrangement. However, as pentosidine is not formed in the absence of oxygen, the latter is

Table 4

Cross-links found in collagen and/or gelatin from skin.

Cross-link	Presence in collagen from skin	Absence in collagen from skin	Presence in gelatin from skin	Formation process
Disulfide bonds	(Bailey & Light, 1989)	n/a ^a	Probably: cleaved in alkaline conditions (Smithies, 1965) n/a	Between cysteine residues
N(γ -glutamyl)lysine peptide	Potentially present in type III collagen (Eyre & Wu, 2005)			Transglutaminase
Aldol cross-link between two lysines	(Baynes & Dominiczak, 2004)	n/a	n/a	Lysyl oxidase pathway
Lysinonorleucine	(Baynes & Dominiczak, 2004)	n/a	n/a	Lysyl oxidase pathway
Hydroxylysinonorleucine (glycosylated or not)	(Eyre & Wu, 2005)	n/a	No: cleaved in acidic conditions (Eyre & Wu, 2005)	Lysyl oxidase pathway
Histidino-hydroxylysinonorleucine (HHL)	(Eyre & Wu, 2005; Robins, 2007; Yamauchi, Woodley, & Mechanic, 1988)	n/a	No: cleaved in acidic conditions (Eyre & Wu, 2005)	Age-related cross-link; Lysyl oxidase pathway
Pentosidine	(Sell et al., 1991; Vos et al., 2013)	n/a	Yes (Van den Bosch & Gielens, 2003)	Advanced glycation endproduct
<ul style="list-style-type: none"> Lysyl – Pyridinoline = Deoxypyridinoline Hydroxylysyl – Pyridinoline = pyridinoline 	(Moriguchi & Fujimoto, 1979; Ricard - Blum, Esterre, & Grimaud, 1993; Robins et al., 2003; Uriarte-Montoya et al., 2011)	(Eyre & Wu, 2005; Ricard - Blum, 2010; Souberbielle, 2000; Yamauchi et al., 1988)	Yes (Uriarte-Montoya et al., 2011; Van den Bosch & Gielens, 2003)	Lysyl oxidase pathway
Desmosine (pyridinium ring)	n/a	(Baynes & Dominiczak, 2004; Ma et al., 2003; Viglio et al., 2000)	Yes (Digenis et al., 1994)	In collagen: Lysyl oxidase pathway In gelatin: Oxydation
Methylene bond	n/a	n/a	Yes (Digenis et al., 1994)	Reaction with aldehydes
Aminal	n/a	n/a	Yes: formed in gelatin but cleaved in acidic conditions (Digenis et al., 1994)	Reaction with aldehydes (pH close to 7)
Aminoglycoside bond (ketose sugar)	n/a	n/a	Yes (Digenis et al., 1994)	Reaction with aldose sugars
<ul style="list-style-type: none"> Lysyl Pyrrol Hydroxylysyl Pyrrol 	(Scott, Qian et al., 1983)	(Eyre & Wu, 2005)	n/a	Lysyl oxidase pathway
Glucosepane	(Monnier et al., 2013; Sjöberg & Bulterijis, 2009)	n/a	n/a	Age-related cross-link; Advanced glycation endproduct
Arginoline	n/a	(Eyre, Weis, & Wu, 2010)	n/a	Lysyl oxidase pathway

^a n/a: no answer.

necessary. Pentoses involved in pentosidine formation could be formed from sugars with more carbons by oxidative fragmentation. Smaller sugars such as trioses, tetroses, and ketoses could also contribute by condensation and/or reverse aldol reactions (Sell et al., 1991).

Pyridinoline and its reduced form (deoxypyridinoline) are two pyridinium ring-type cross-links (Robins et al., 2003) formed by the lysyl oxidase pathway (Ricard-Blum, 2010; Samma et al., 1996) (Table 3). These cross-links are naturally located in non-helical regions of collagen (called the telopeptide region) at the extremity of the triple-helices (Souberbielle, 2000). Pyridinoline and deoxypyridinoline are also referred in the literature as hydroxylysyl-pyridinoline and lysyl-pyridinoline, respectively (Ricard-Blum, 2010; Samma et al., 1996). The existence of different names for the same cross-links complicates their inventory. Moreover, the presence of pyridinoline and deoxypyridinoline in collagen from skin is still controversial (Table 4).

In a second part the covalent bonds found in gelatin are described (Table 4). As gelatin results from the collagen denaturation, some cross-links described above remain while other covalent bonds are formed due to chemical and environmental conditions during and after the manufacturing process. These cross-links are favoured by high temperature and humidity but also by UV-light and chemical compounds like formaldehyde and reducing sugars (Singh, Rama Rao, Venugopal, & Manikandan, 2002).

The different mechanisms assumed to be responsible for cross-links formation in gelatin have been described by Digenis, Gold, and Shah (1994). During oxidation, a lysine residue is deaminated and its free amine function is replaced by an aldehyde group. This group is then attacked by another free amine function of an adjacent lysine residue and an imine bond is created. Several aldol type reactions between the imine and other lysines give a desmosine-type cross-link (Fig. 8). According to the authors, desmosine are pyridinium rings which are different from the pyridinoline described by Ricard-Blum et al. (2010) although desmosines and pyridinolines come from the same pathway, i.e. the allysine pathway. Indeed, in this pathway (in the extracellular matrix), the lysyl oxidase deaminates a lysine residue to give an allysine. When several allysines are close to each other, they interact to form a pyridinium ring (desmosine-type cross-link). However, so far desmosine was not found in collagen while it was reported in elastin (Baynes & Dominiczak, 2004; Ma, Lieberman, Turino, & Lin, 2003; Viglio et al., 2000).

Other cross-links have been found in gelatin and the underlying mechanisms of their formation characterized. A free amine group of a lysine residue may react with an aldehyde group. This reaction gives a hydroxymethylamine which yields a molecule of water to create a secondary aldimine. This imine reacts with another lysine residue also changed into a hydroxymethylamine to give dimethylene ether. This compound undergoes rearrangements to form a methylene bond between two lysine residues (Fig. 9). The

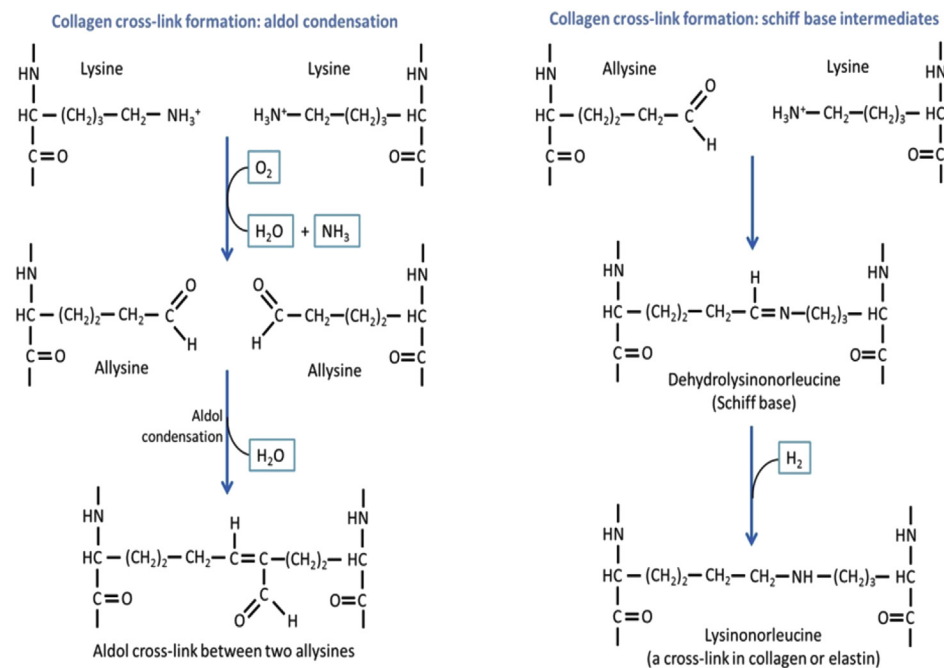


Fig. 5. Two covalent bonds formed by the allysine pathway according to Baynes and Dominiczak (2004).

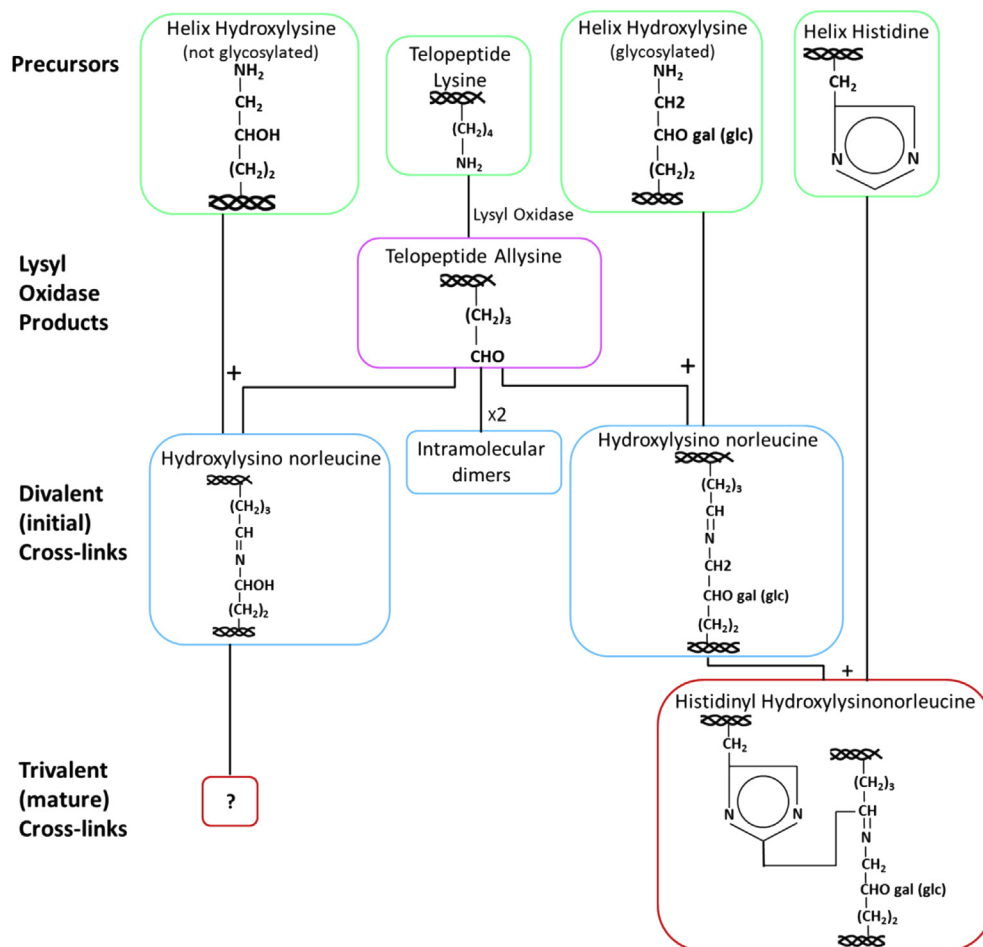


Fig. 6. Allysine cross-linking pathway (adapted from Eyre and Wu (2005)).

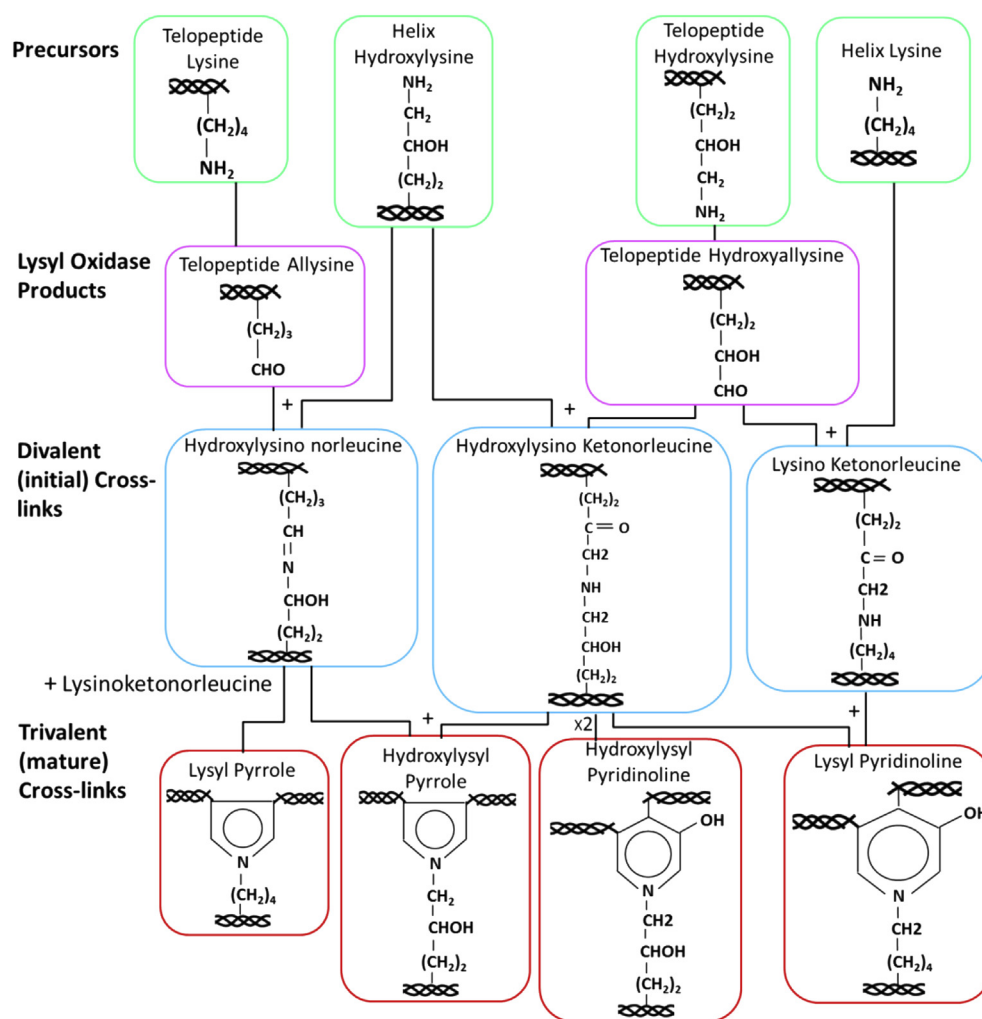


Fig. 7. Allysine cross-linking pathway (adapted from Eyre and Wu (2005)).

cationic imine created in the previous scheme (Fig. 9) may also react with a free amino group of an amino acid to create an aminal (the amine form of an acetal). The pH plays an important role in this reaction. Indeed, under acidic conditions, the first step of the reaction is rate limiting and the second step becomes rate limiting in basic pH. This explains why the optimal pH is close to 7 (Fig. 10). This aminal formation may occur between lys-lys, arg-lys and arg-arg amino acids. When lysine and arginine residues are in the presence of formaldehyde, this reaction gives a lysine-arginine aminal. During the drying process of a cross-linked gelatin with formaldehyde, this kind of reaction may also occur between two arginine residues to give an arginine-arginine aminal cross-link (Fig. 11). The humidity rate plays an important role in the formation of this latter covalent bond (Digenis et al., 1994). However, there is no information about the humidity threshold necessary to induce this reaction. Sugars like glucose and other aldoses comprise an aldehyde group. The latter may react with a free amino group of an amino acid to create an imine. This imine undergoes several rearrangements to become a ketose that creates a covalent bond with an amino group of an amino acid and its carbonyl function (Fig. 12). In gelatin these types of cross-link may occur between lys-lys, arg-lys and arg-arg residues which present free amino groups (Digenis et al., 1994).

Cross-links in collagen and therefore in gelatin present various forms and imply not only lysine residues but also arginine, histidine

and methionine (disulfide bonds) and sugars. They occur in an intra or inter-molecular way and can be located at the extremity of helical regions as well as inside triple-helices. Regarding the literature, the presence of pyridinoline cross-links in skin is still under discussion.

4.3. Parameters affecting cross-link formation

As described previously, cross-linking reactions imply the presence of aldehyde groups, imine or ketones. These groups are not only naturally present in raw material such as skin but also in drugs contained in pharmaceutical capsules or they can be added during the capsules manufacturing process. Chemical compounds which have been identified as favoring cross-link formation are aldehydes, imines, ketones, saccharides (glucose and aldose sugars), dyes (FD&C Red No. 3 or 40 and Blue No. 1), calcium carbonate, hydrogen peroxide, sulfonic acids and p-toluene sulfonic acid, carbodiimides (1-ethylene 3-(3-dimethylamino propyl) carbodiimide hydrochloride, guanidine hydrochloride), benzene (benzene derivatives are used in drugs production and benzene itself is still used in Asia (Auger, 2003; Bemis & Murcko, 1996) and terephthaloyl chloride (Singh et al., 2002)).

Other chemical compounds used in drugs can be transformed into reactive compounds as it is the case for

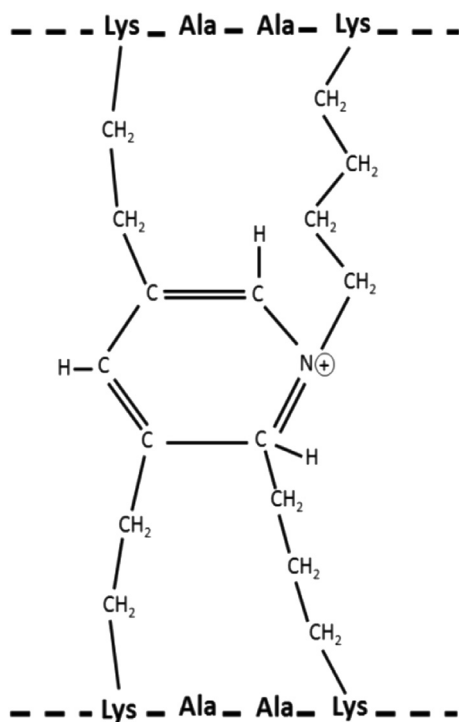


Fig. 8. Pyridinium ring in gelatin adapted from Digenis et al., (1994). Copyright (1994) Wiley. This material is reproduced with permission of John Wiley & Sons, Inc.

hexamethylenetetramine (a stabilizer) which is transformed, under humid conditions, into ammonia and formaldehyde. This stabilizer indirectly induces cross-links in gelatin by forming aldehyde in high level humidity conditions. Humidity may play a role as a catalyzer in the formation of imines, which is the origin of the covalent bonds described previously. Moreover, the decrease of humidity may increase the level of arginine bonds in gelatin already cross-linked with formaldehyde. Several studies on HGC filled with drugs or not, shown a decrease of dissolution degree (in deionized water) when stored at high relative humidity and temperature conditions (Digenis et al., 1994). The authors provided no information on the exact humidity rate necessary to raise the level of cross-linking in gelatin or on the mechanisms involved in cross-link formation with humidity.

High temperature condition also increase the level of cross-linking. Heating dried gelatin to 105 °C should lead to the formation of cross-links between free amino groups of amino acids and thus to its insolubilization (Digenis et al. 1994; Yannas and Tobolosky (1967)). However, no reduction in the level of the free amino group in gelatin stored at elevated temperatures was observed, despite the reduction of the dissolution level. It was concluded that molecular bonds other than the free amino group cross-links are caused by temperature (Ofner et al., 2001). Although the temperature has been identified as a factor increasing cross-links and reducing gelatin dissolution, the mechanisms involved in this phenomenon are still unknown. Light and particularly UV-light also increase cross-link formation in gelatin (Rabotyagova, Cebe, & Kaplan, 2008). Karim and Bhat (2009) have shown that gel strength increased under UV rays because of the cross-links formed between helices in gelatin. Bessho, Kojima, Okuda, and Hara (2007) reported that insolubility due to the crosslinking of gelatin hydrogels was caused by UV light at doses above 8 kG gamma-irradiation. UV light is used to form bonds in gelatin to increase gel stability and melting temperature. It is more for pharmaceutical capsules and it is an ecological alternative to the

use of chemical compounds (like glutaraldehyde) to induce cross-linking (Yamamoto, Koike, & Dobashi, 2007).

5. Mechanisms and factors influencing dissolution

Gelatin dissolution in water is performed in two steps: first, the gelatin swells and then melts when the melting temperature is reached. The swelling and melting steps are influenced by various external factors such as pH, ionic strength and water temperature.

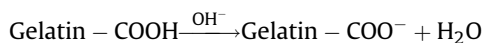
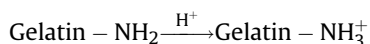
5.1. Effect of the physicochemical environment on gelatin swelling behavior

Despite its wide range of use, the structure of gelatin and its dissolution and swelling mechanisms have been little investigated. However, some publications have demonstrated that swelling and dissolution depend on the pH, temperature and salt concentration of the solvent (Gordon, Brooker, Chew, Wilson, & York, 2010; Mercade-Prieto, Sahoo, Falconer, Paterson, & Ian Wilson, 2007; Yang et al., 1997).

Gordon et al. (2010) investigated the swelling of gelatin films prepared from supermarket leaf pork gelatin using the scanning fluid dynamic gauge technique (SFDG). The exact origin of the gelatin (skin or bones) was not specified. They showed that the equilibrium of swelling was not reached after 2 h in reverse osmosis (RO) water at pH 5 and 20 °C and that equilibrium time was strongly dependent on swelling conditions. When equilibrium was reached, the authors demonstrated that the increase of water temperature above 20 °C had a considerable influence on the swelling of gelatin (the thickness of gelatin films increased with temperature from 20 °C to melting temperature: around 27 °C). Under 20 °C, the swelling temperature had little effect on film thickness.

The effect of pH on swelling behavior was also investigated by Gordon et al. (2010). Swollen samples in reverse osmosis (RO) water were placed in a solution of varying pH. When the pH value was under the pKa of the amine function of both proline and hydroxyproline (10.6), the films did not swell but shrank as pH increased. The increase of the solution's ionic strength as the pH increased caused water to move from the gel to the solution (because the gels contained no salts). Above the pKa value, the films swelled considerably because of the repulsion of charged protein chains. This shrinkage phenomenon was not observed when the gelatin films were first swelled in water containing 0.01 M NaCl (Gordon et al., 2010). These results confirmed that both ionic strength and pH played an important role in the swelling behavior of dried gelatin films (Mercade-Prieto et al., 2007).

In gelatin already cross-linked with 2% formaldehyde, Yang et al. (1997) showed that swelling behavior was a function of pH and salt concentration. Indeed, the gels shrank when pH was close to the isoelectric point of gelatin gels (5.5) and swelled when pH changed. But the swelling behavior was asymmetric with respect to the isoelectric point, as shown in Fig. 13(a) which indicates that the cationic gels (pH < pHi) tended to swell more than the anionic ones (pH > pHi). A variation of pH changes the charge of the gelatin with the amino and carboxyl groups, so the hydrogel evolves to a cationic or anionic one:



The swelling of a gelatin gel decreases when increasing NaCl content. This phenomenon may be attributed to the formation of

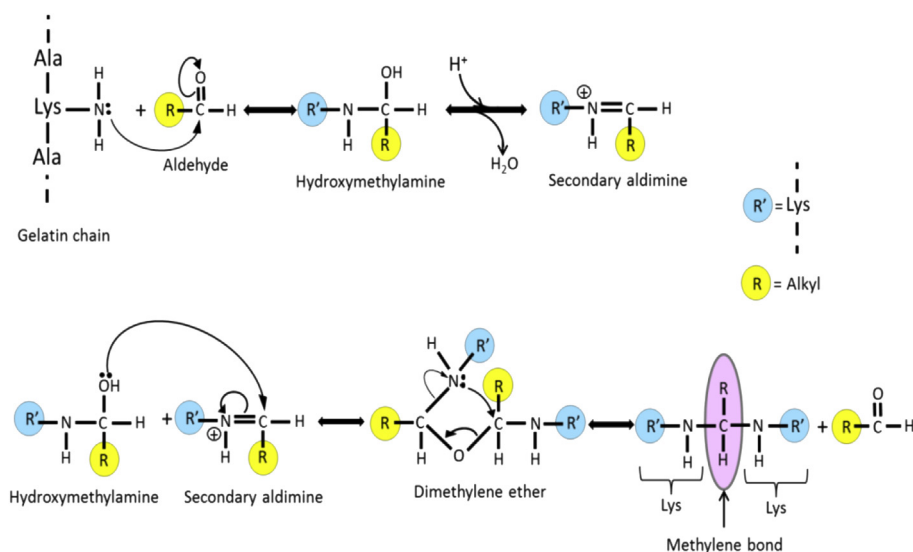


Fig. 9. Methylene bond formation in gelatin adapted from Digenis et al., (1994). Copyright (1994) Wiley. This material is reproduced with permission of John Wiley & Sons, Inc.

ion pairs between network charges and ions in solution (Yang et al., 1997).

Yang et al. (1997) showed that all the hydrogels collapsed as the NaCl concentration increased, except for the hydrogel at pHi (Fig. 13, b). They attributed this observation to the antipolyelectrolyte swelling behavior of ampholytic hydrogels described by Huglin and Rego (1991). Indeed, the opposite charges in the gel present a high level of attraction in low-ionic-strength media. This leads to a collapse of the gel network. With the addition of salt, the attractive interactions inside the gel are screened and the ionic bonds are destroyed. The polymer formed electrostatic interactions with the solvent and the hydrogel network swelled (Yang et al., 1997).

5.2. Parameters influencing solubility

5.2.1. Origin of raw material and concentration

Nur Hanani, Roos and Kerry (2012) studied the dissolution of different gelatin films from beef, pork and fish skins. They observed that the degree of dissolution of gelatin depends on the raw material and sometimes on gelatin concentration. Indeed, contrary to beef and fish gelatin, the solubility of pig skin gelatin rose as its concentration increased (from 4% to 6 and 8%). Fish gelatin films presented a better degree of dissolution than both beef and pig gelatin films. The latter exhibited the lowest dissolution degree.

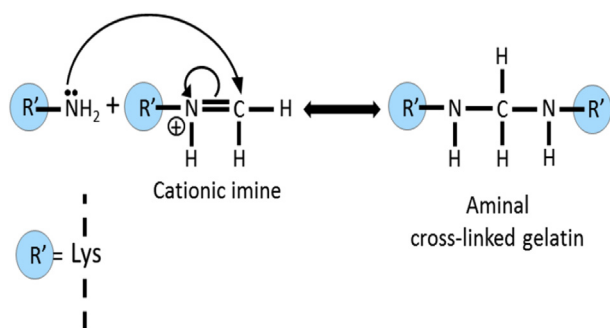


Fig. 10. Amination mechanism formation in gelatin adapted from Digenis et al., (1994). Copyright (1994) Wiley. This material is reproduced with permission of John Wiley & Sons, Inc.

The content of imino acids Pro and Hyp constitutes the main difference between fish and mammalian gelatin. These imino acids stabilize the structures during gel formation. Cold-water-fish gelatin contains fewer imino acids Pro and Hyp than gelatin from warm-water-fish, which is close to mammalian gelatin (Haug et al., 2003). The lower content of Hyp and Pro in cold-water-fish gelatin probably explains its lower gel modulus, gelling and melting temperature, and lower thermal stability than mammalian gelatin (Grossman & Bergman, 1992).

5.2.2. High molecular weight

The dissolution of gelatin depends on its degree of cross-linking. Digenis et al. (1994) described the different possible cross-links in gelatin and established a relationship with drug delivery. Indeed, when gelatin is exposed to factors which increase the degree of cross-linking (see part 4.3), the dissolution value decreased. According to Ofner et al. (2001) and Welz and Ofner (1992), cross-linking gelatin leads to an intricate network of high molecular weight that produced a swellable hydrogel but substantially reduced, or even prevented, the dissolution of the gelatin.

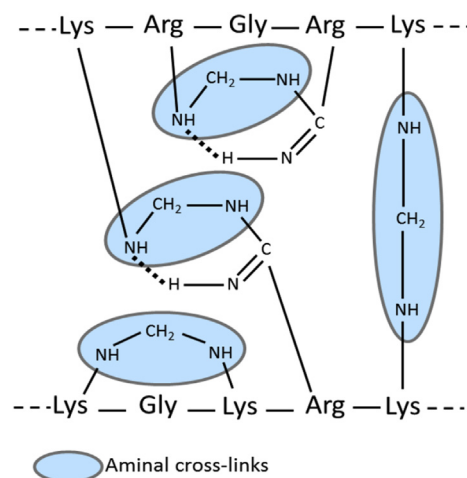


Fig. 11. Amination cross-links found in gelatin adapted from Digenis et al., (1994). Copyright (1994) Wiley. This material is reproduced with permission of John Wiley & Sons, Inc.

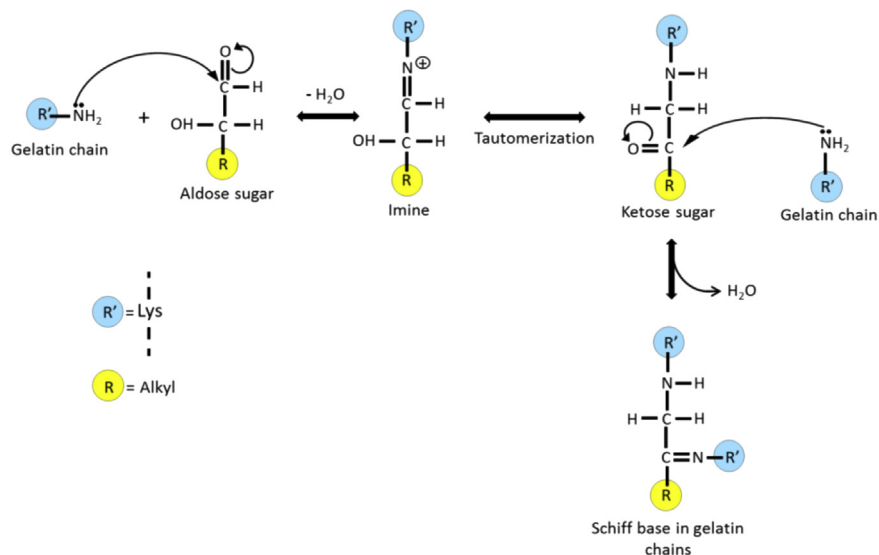


Fig. 12. Cross-link formation between a ketose and an amino group in gelatin adapted from Digenis et al., (1994). Copyright (1994) Wiley. This material is reproduced with permission of John Wiley & Sons, Inc.

Molecular weight distribution in gelatin is different according to the raw material used. Fish gelatins have slightly lower molecular weights than those present in porcine gelatin (Chiou et al., 2006; Muyonga et al., 2004). According to these previous

observations (on raw material and molecular weights), the higher degree of high molecular weights in mammalian gelatin is correlated with the higher contents of amino acids involved in cross-link formation. All these observations correlate the

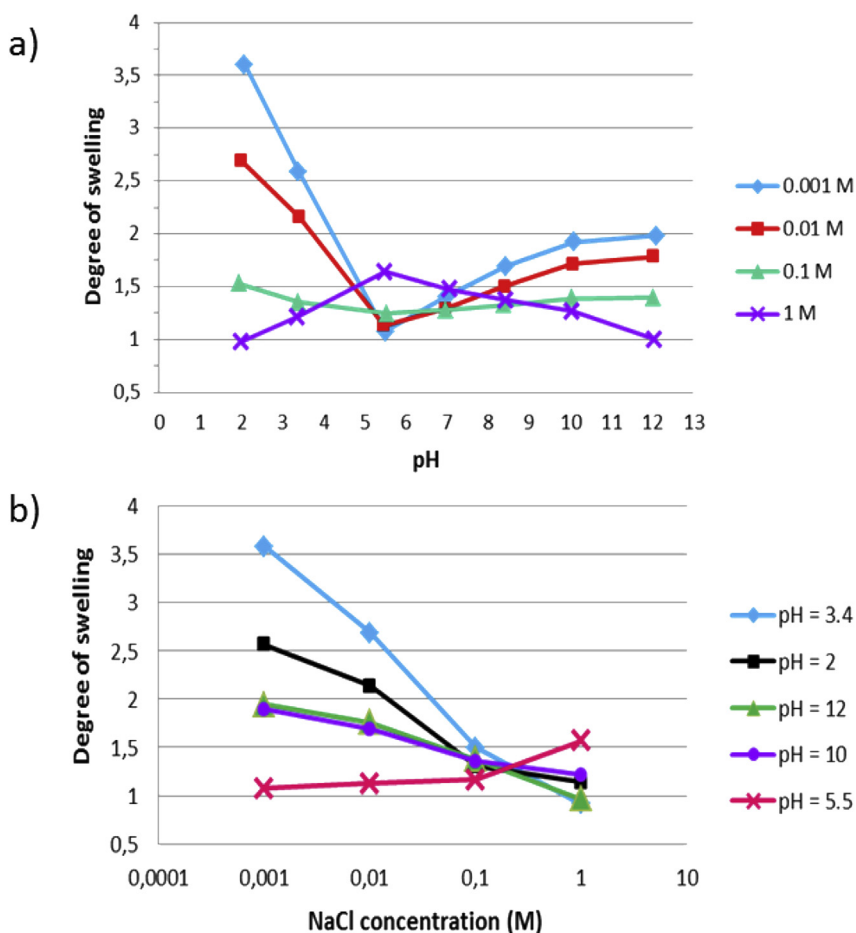


Fig. 13. Degree of swelling of gelatin gels in different NaCl aqueous solutions as a function of pH (a) and as a function of NaCl concentration at different pH values (b) (after Yang et al. 1997).

implication of cross-link formation with high molecular weights. Moreover, Haug et al. (2003) and Grossman and Bergman (1992) confirmed the influence of imino acid content on the increase of the gelatin melting temperature. This corroborates the fact that high molecular weights increase melting temperature and therefore reduce gelatin solubility (Elharfaoui et al., 2007; Ofner et al., 2001; Welz & Ofner, 1992).

High molecular weight compounds may be formed by cross-linked gelatin molecules that could form aggregates in gelatin gel. Indeed, Tromp, Ten Grotenhuis, and Olieman (2001) studied the aggregation of different gelatins above gelling temperature using Size Exclusion Chromatography combined with Multi-Angle Laser Light Scattering SEC-MALLS. They observed compounds with high molecular weights and rich in aggregates which were different from the triple-helical structures usually found in gelatin.

5.2.3. Aggregates

There is no definition of aggregates in gelatin. Cross-links in gelatin could form aggregates of high molecular weight that decrease the dissolution of dried gelatin in water (Rbii, 2010). These high molecular weight aggregates are assumed to be formed in old or degraded gelatin under high humidity, UV light and temperature conditions in storage or when exposed to chemical compounds. Thus the aggregates are formed and stabilized by cross-links. However, a recent publication by Xu et al. (2012) dealt with the aggregation of gelatin grafted with glycidol and showed that aggregates may also be formed by other kind of bonds. Aggregates were formed in gelatin at increasing concentration. The size and the shape of aggregates were studied by Environmental Scanning Electron Microscopy (ESEM) which revealed irregular aggregates at a concentration of 2% (w/w). After increasing the gelatin concentration, several spindle aggregates appeared around 2 μm in size at 6% (w/w) gelatin (Fig. 14).

Then, at higher concentrations the authors observed changes in the shape and size of aggregates (irregular, network or butterfly aggregates). The spindle-shaped aggregates reappeared at 14% (w/w) gelatin. Although the shape was quite similar to the morphology of the aggregates at 6%, the size of the latter increased with concentration and reached 3–4 μm . This large scale structure is an unfavorable factor for chemical reaction. The authors explained all these observations by hydrophobicity in some regions of the gelatin network, which may be the cause of packing. These variations of aggregate morphology and size can be caused by changes due to the increase of concentration. However, as the gelatin was grafted with

glycidol and as glycidol contains epoxy groups which react with $-\text{NH}_2$ groups of gelatin chains, these observations may not reflect the real impact of gelatin concentration on aggregate formation. Indeed, Xu et al. (2012) studied the effect of glycidol on gelatin and showed that grafting density in gelatin reached a peak value at concentrations of 6% and 14% which correspond to the observation of the spindle aggregates. However the authors did not explain the effect of the potential cross-links formed by glycidol with the $-\text{NH}_2$ groups of gelatin chains on these aggregates. They carried out analyses to characterize the morphology of aggregates more precisely on the basis that they were formed and stabilized by weak bonds.

The UV spectroscopy analysis indicated that, with an increase in gelatin concentration, hydration of the network is partly destroyed and the hydrophobic interactions between gelatin chains become stronger. Thus chains can easily form intra or intermolecular hydrogen bonding rather than with the solvent. According to the UV results, hydrophobic interactions increased and competed with hydrogen bonding at increasing concentration. Electrostatic repulsions in the hydrophobic regions are considerable and lead to the formation of a β -sheet structure. The circular dichroism (CD) spectrum of gelatin confirmed these results showing that the network evolves from a random coil state to a β -sheet or other secondary structure when concentration is raised. Indeed, the authors observed a characteristic peak of β -sheets which appeared when gelatin concentration increased.

To confirm that hydrophobic and hydrogen bonds play a key role in aggregates formation, Xu et al. (2012) added urea in samples which inhibited hydrogen bonds, and SDS which changed the hydrophobic interactions. The spindle aggregates observed at 6% concentration were destroyed under the action of urea. In the presence of SDS, aggregates were formed and were larger. The authors concluded that hydrophobic interactions have an influence on the diameter of aggregates and hydrogen bonds influence their formation. Variations of pH and NaCl concentrations caused changes in the shape of the aggregates, indicating that electrostatic interactions imply gelatin chain arrangements in aggregates.

Xu et al. (2012) identified aggregate structures as β -sheets that formed with the increase of gelatin concentration by hydrogen and hydrophobic bonds. However, the authors studied gelatin grafted with glycidol which reacts with $-\text{NH}_2$ groups of gelatin. Moreover, no other publications report the observation of β -sheets structures in gelatin chains. Coppola et al. (2012) observed that gelatin in crystallized form was composed by bundles of triple-helices. These bundles of triple-helices could also be considered as aggregates in gelatin.

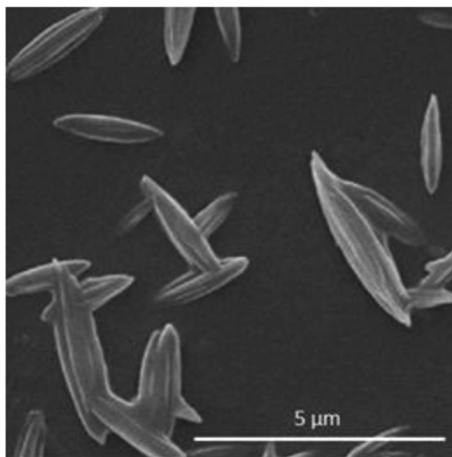


Fig. 14. Example of aggregates in a 6% (w/w) gelatin grafted with glycidol observed by Xu et al. (2012) by ESEM.

5.2.4. Impact of the manufacturing process

During the manufacturing process, the thermal extraction of gelatin causes Maillard reactions. These reactions involve amine groups and carbonyl groups which form Schiff bases and lead to various complex compounds as a function of the reaction pathway (Fig. 15) (Martins, Jongen, & van Boekel, 2000). A brown coloration of the gelatin extract, linked to these Maillard reactions, appears during the extraction step and its intensity depends on the temperature. Maillard reactions could reduce gelatin solubility through the formation of cross-links (Rbii, 2010).

5.2.5. Dissolution test

The dissolution test in deionized water has been criticized as there are enzymes in gastric fluids which participate in the dissolution of hard gelatin capsules (Meyer et al., 2000). Another test (the two-tier test) has been developed which allows the use of enzymes in the dissolution media (Cole et al., 2008). According to St Clair, Purdie, Hu and McGeoch (2010), this two-tier test presents the same dissolution rates as the dissolution test in water with hard

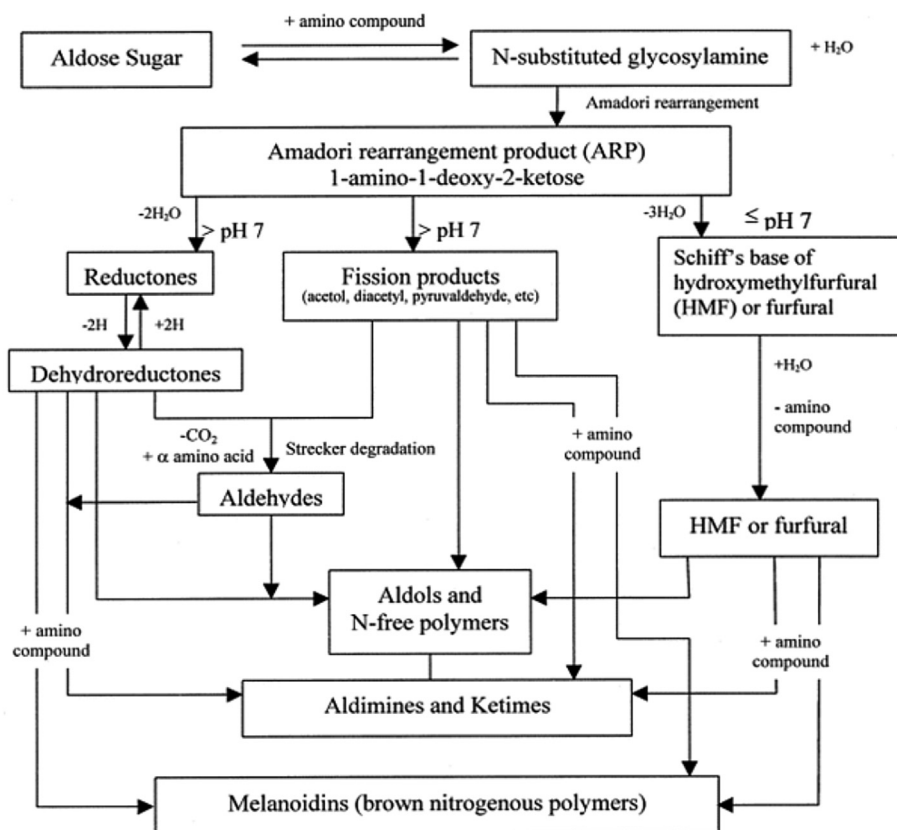


Fig. 15. Maillard reactions scheme (Martins et al., 2000).

gelatin capsules (HGC) with a high degree of cross-linking. HGCs with a high level of cross-linking present a sufficient dissolution rate if the media contains higher quantities of enzyme. However, the correlations with the *in vivo* concentrations of enzymes still need to be established (St Clair et al., 2010). The impact of insufficient solubility in water on the release of drugs in the organism has not been established as it has in gastric fluids, in which HGCs present a sufficient dissolution rate. According to Digenis et al. (1994), doubts have been raised on the significance of the dissolution test conducted in an enzyme-free medium compare to the *in vivo* performance of compounds encapsulated in HGC. Zeng (2010) explained that some *in vitro* dissolution curves presented a delay of few minutes in initial dissolution. This observation may be interpreted as a non-sufficient dissolution rate of these gelatins whereas it is due to a delay in initial dissolution. Even if this *in vitro* delay of dissolution would be found in *in vivo* conditions, it would not have any significance (Zeng, 2010).

6. Conclusion

Gelatin is a complex biopolymer and its dissolution is influenced by many parameters. Although it has been used throughout history, its structure and composition and the impact of various factors on its solubility have not been determined with precision. Cross-links are the factor most studied and involved in the decrease of gelatin dissolution. However, the number and type of cross-links in gelatin and in collagen are still subject to debate. Cross-links can form high molecular weight compounds. The latter are not well identified. Some authors have described aggregates in gelatin as having high molecular weights, but the definition of an aggregate is still not clear. It seems that cross-links lead to high molecular weights which can result in aggregates. These aggregates are stabilized by

weak bonds like hydrogen and hydrophobic interactions, but their molecular structures are still not known. They could be bundles of triple-helices or β -sheet structures. The latter structure was suggested by Xu et al. (2012) and must be confirmed by further experiments since it was established on gelatin grafted with glycidol and not on raw gelatin. Regarding the literature, aggregates (in raw gelatin) are more probably made of bundles of triple-helices than of β -sheet structures. The type and localization of weak bonds in aggregates is not known.

Among all the factors identified as influencing the dissolution of gelatin, no hierarchy of their importance has yet been established. Cross-links were given more attention in this article but the structure of gelatin chains could also have an influence on gelatin solubility. The question is which has the larger impact on gelatin dissolution: cross-links or the spatial arrangement of molecules?

Environmental factors like temperature, UV light and humidity rate are often involved in increased cross-link formation. However, there is no specification of the time and rate of exposure necessary to produce cross-link formation. Although humidity is assumed to catalyze imine formation and increase the level of arginine bonds, there is little knowledge on which cross-link is created by the influence of environmental factors. The mechanisms of cross-link formation induced by these factors are not known.

The exact composition of pig skin gelatin can be also discussed because, according to Farris et al. (2009), there is no cysteine in pig skin gelatin but cysteine is found in type III collagen, a collagen found in skin (Bailey & Light, 1989). Cysteine creates disulfide bonds in collagen and if this amino acid is found in pig skin, it could be responsible for the disulfide bonds in gelatin. The composition of this polymer is often reduced to the type and amount of amino acids which compose the collagen molecules. However, as gelatin is

extracted from animal tissues by thermal treatments, sugars, lipids and other proteins can also be found.

The issue of the dissolution of hard gelatin capsules is difficult to understand due to the high complexity of gelatin and the many parameters identified as potentially involved in its solubility. It is even more complex given the variability of the results obtained by current *in vitro* dissolution tests.

In the future, an effort should be made to link structural and compositional properties of gelatin with its dissolution degree such as dynamic light scattering to study the size of aggregates or spectroscopy techniques (fluorescence, infra-red or raman) to get new insights in the chemical functions involved in the structural properties.

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